Amendment to the Specification

Attachment: Appendices G and H

Please replace paragraphs [0014]–[0024] with the following amended paragraphs:

~~**[0014]** *Figure 2* gives a representative list of accession codes for various TMV genome sequences.~~

[0015] ~~*Figure 3*~~ *Figure 2* presents a schematic representation of certain families of viruses that infect plants.

~~**[0016]** *Figure 4* presents accession codes for a variety of A1MV genome sequences.~~

Please replace paragraph [0017] with the following amended paragraph:

[0017] ~~*Figure 5*~~ *Figure 3* shows a Western blot of protoplasts infected with *in vitro* transcripts of Av/A4, an A1MV-based vector employed in certain studies described herein (Spitsin, S., et al., *Proc. Natl. Acad. Sci.* 96(5): 2549-2553, 1999). Samples were analyzed 24 hours post inoculation. C- is a negative control. The arrow indicates an A1MV CP band detected by A1MV CP-specific monoclonal antibodies.

[0018] ~~*Figure 6*~~ *Figure 4* shows pepper plants and *Nicotiana benthamiana* plants infected with wild type A1MV.

[0019] ~~*Figure 7*~~ *Figure 5* is a Western blot of N *benthamiana* plants infected with *in vitro* transcripts of Av/A4. Samples were analyzed 12 days post inoculation. C- is extract from healthy plants. The arrow points to A1MV CP bands detected by A1MV CP-specific monoclonal antibodies.

[0020] ~~*Figure 8*~~ *Figure 6* presents a schematic diagram of the genomic organization of 125C (~~Figure 8A~~ Figure 6A) and D4 following insertion of a polynucleotide of interest (~~Figure 8B~~ Figure 6B). The 126/183 kDa protein is required for replication of the virus. The MP is the movement protein that mediates cell-to-cell movement. Arrows indicate positions of the subgenomic promoter. The shaded region represents TMV coat protein sequences that contain a *cis* element that may be required for optimal replication. The black box represents a polynucleotide of interest, e.g., a foreign gene.

[0021] ~~*Figure 9*~~ *Figure 7* shows a Western blot of protoplasts infected with *in vitro* synthesized transcripts of 125C/hGH (125C as shown in ~~Figure 8A~~ Figure 6A, in which the foreign gene encodes hGH). Samples were analyzed 24 hours post inoculation. 1 ug of purified hGH was loaded as a standard.

[0022] ~~*Figure 10*~~ *Figure 8* is a Western blot showing detection of hGH in N *benthamiana* plants 11 days post infection (dpi).

[0023] ~~*Figures 11a-11d*~~ *Figures 9a-9d* presents schematics of various D4-related vectors. 126/183 kDa are the replicase proteins, MP is the movement protein required for cell-to-cell movement. Nucleotide numbers represent positions in the wild type TMV genome. C3GFP is the cycle3 mutant of green fluorescent protein (GFP) (Crameri A, Whitehorn EA, Tate E, Stemmer WP, Nat Biotechnol., 14(3): 315-9, 1996). The asterisk indicates mutated C3GFP in which the *Nco*I site and the *Xho*I sites in the ORF have been eliminated by mutation using PCR. *Pst*I-*Xho*I sites were used to introduce sequences from A1MV RNA3 that include the origin of assembly (OAS).

[0024] ~~*Figures 12a-12e*~~ *Figures 10a-10c* show pictures of infected plants, demonstrating that A1MV complements D4GFP, which does not have a functional coat protein coding sequence and is limited in systemic spread, and facilitates its movement throughout the plant. ~~*Figure 12a*~~ *Figure 10a* shows a picture of a plant that was co-inoculated with SR27 (a TMV-based vector lacking CP coding sequence and including a GFP transgene under control of the subgenomic CP promoter) and A1MV. The image (taken under UV light) demonstrates spread of virus into the upper uninoculated leaves. ~~*Figure 12b*~~ *Figure 10b* (taken under UV light) shows a picture of a plant that was inoculated with SR27 only. Lack of fluorescence in the upper leaves indicates that virus infection was limited to locally inoculated leaves. ~~*Figure 12c*~~ *Figure 10c* shows the same plant as in ~~Figure 12a~~ *Figure 10a*, under normal light.

Please replace paragraph [0034] with the following amended paragraph:

[0034] As noted above, the present invention provides systems for expressing a polynucleotide or polynucleotides of interest in plants. In preferred embodiments, these systems include one or more viral vector components. A wide variety of viruses are known that infect various plant species, and can be employed for polynucleotide expression according to the present invention. ~~Figure 3~~ Figure 2 presents a schematic representation of certain families of viruses that infect plants. Appendix A provides a representative list of plant virus families, based on the type of nucleic acid (e.g., dsDNA, ssDNA, ssRNA, dsRNA, or unassigned) that makes up the viral genome. Additional information can be found, for example, in *The Classification and Nomenclature of Viruses*, Sixth Report of the International Committee on Taxonomy of Viruses" (Ed. Murphy et al.), Springer Verlag: New York, 1995, the entire contents of which are incorporated herein by reference (see also, Grierson et al., Plant Molecular

Biology, Blackie, London, pp. 126-146, 1984; Gluzman et al., *Communications in Molecular Biology: Viral Vectors*, Cold Spring Harbor Laboratory, NY, pp. 172-189, 1988; Mathew, *Plant Viruses Online* ~~(http://imagels.uidaho.eduivide/)~~.

Please replace paragraph [0093] with the following amended paragraph:

[0093] A1MV is an *Alfamovirus*, closely related to the *Ilarvirus* group and is a member of the *Bromoviridae* family. The genome of A1MV consists of three positive-sense RNAs (RNAs 1-3) (See ~~Figure 4~~ Appendix H, which presents accession codes for a variety of A1MV genome sequences). RNAs 1 and 2 encode replicase proteins P1 and P2, respectively; RNA3 encodes the cell-to-cell movement protein P3. A subgenomic RNA, RNA4, is synthesized from RNA3. This subgenomic RNA4 encodes the viral coat protein (CP). CP participates in viral genome activation to initiate infection, RNA replication, viral assembly, viral RNA stability, long-distance movement of viral RNA, and symptom formation. A1MV depends on a functional P3 protein for cell-to-cell movement, and requires the CP protein throughout infection. Depending on the size of the CP-encapsidated viral RNA, virions of A1MV can vary significantly in size (e.g., 30- to 60-nm in length and 18 nm in diameter) and form (e.g., spherical, ellipsoidal, or bacilliform). The host range of A1MV is remarkably wide and includes the agriculturally valuable crops alfalfa (*Medicago sativa)*, tomato (*Lycopersicon esculentum*), lettuce (*Lactuca sativa*), common bean (*Phaseolus vulgaris*), potato (*Solanum tuberosum*), white clover (*Trifolium repens*) and soybean (*Glycine max*). Particular susceptible host species include, for example, *Abelmoschus esculentus, Ageratum conyzoides, Amaranthus caudatus, Amaranthus retroflexus, Antirrhinum majus, Apium graveolens, Apium graveolens* var. *rapaceum, Arachis hypogaea, Astragalus glycyphyllos, Beta vulgaris, Brassica campestris* ssp. *rapa, Calendula officinalis, Capsicum annuum, Capsicum frutescens, Caryopteris incana, Catharanthus roseus, Celosia argentea, Cheiranthus cheiri, Chenopodium album, Chenopodium amaranticolor, Chenopodium murale, Chenopodium quinoa, Cicer arietinum, Cichorium endiva, Coriandrum sativum, Crotalaria spectabilis, Cucumis melo, Cucumis sativus, Cucurbita pepo, Cyamopsis tetragonoloba, Daucus carota* (var. sativa), *Dianthus barbatus, Dianthus caryophyllus, Emilia sagittata, Fagopyrum esculentum, Gomphrena globosa, Helianthus annuus, Lablab purpureus, Lathyrus odoratus, Lens culinaris, Linum usitatissimum, Lupinus albus, Macroptilium lathyroides, Malva parviflora, Matthiola incana, Medicago hispida, Melilotus albus, Nicotiana bigelovii, Nicotiana clevelandii, Nicotiana debneyi, Nicotiana glutinosa, Nicotiana megalosiphon, Nicotiana rustica, Nicotiana sylvestris, Nicotiana tabacum, Ocimum basilicum,*

Petunia x hybrida, Phaseolus lunatus, Philadelphus, Physalis floridana, Physalis peruviana, Phytolacca americana, Pisum sativum, Solanum demissum, Solanum melongena, Solanum nigrum, Solanum nodiflorum, Solanum rostratum, Sonchus oleraceus, Spinacia oleracea, Stellaria media, Tetragonia tetragonioides, Trifolium dubium, Trifolium hybridum, Trifolium incarnatum, Trifolium pratense, Trifolium subterraneum, Tropaeolum majus, Viburnum opulus, Vicia faba, Vigna radiata, Vigna unguiculata, Vigna unguiculata ssp. sesquipedalis, and *Zinnia elegans.*

Please replace paragraph [0095] with the following amended paragraph:

[0095] The TMV genome is 6395 nucleotides long and is encapsidated with a 17.5 kD CP, which produces 300 nm-long rods. In addition to CP, TMV has three nonstructural proteins: 183 and 126 kD proteins are translated from genomic RNA and are required for viral replication. The 30 kD movement protein provides for the transfer of viral RNA from cell-to-cell. A representative list of accession codes for TMV genome sequence information is included ~~as Figure 2~~ in Appendix G; Appendices B-F show sequence alignments for the tobamovirus helicase, RNA-dependent RNA polymerase (a replicase), movement protein, coat protein, and methyltransferase genes, respectively, from various tobamoviruses. Plant species susceptible to infection with TMV include *Beta vulgaris, Capsicum frutescens, Chenopodium amaranticolor, Chenopodium hybridum, Chenopodium quinoa, Cucumis melo, Cucumis sativus, Cucurbita pepo, Datura stramonium, Lactuca sativa, Lucopersicon esculentum, Lycopersicon pimpinellifolium, Nicotiana benthamiana, Nicotiana bigelovii, Nicotiana clevelandii, Nicotiana debneyi, Nicotiana glutinosa, Nicotiana rustica, Nicotiana sylvestris, Nicotiana tabacum, Papaver nudicaule, Phaseolus vulgaris, Physalis floridana, Physalis peruviana, and Solanum tuberosum.*

Please replace paragraph [0098] with the following amended paragraph:

[0098] We have constructed a vector based on the Tobacco Mosaic Virus that is adapted for insertion of a polynucleotide of interest to generate a producer vector according to the present invention. Specifically, we have generated vectors that are deficient in CP production (see ~~Figures 8 and 11~~ Figures 6 and 9; vector D4 is represented with a generic polynucleotide inserted; vector SR-27 and related vectors are derived from D4 as described further in Example 4). We have demonstrated that infection with such vectors is limited to locally inoculated leaves. These vectors depends upon a second vector for systemic movement.

Please replace paragraphs [00100]–[00103] with the following amended paragraphs:

[00100] An A1MV-based vector referred to as Av/A4, which contains a functional A1MV coat protein gene, has been constructed. As shown in ~~Figure 5~~ Figure 3, we have established a tobacco protoplast system and tested the components of this vector. Depicted is a Western blot showing accumulation of virus coat protein, indicating infection of protoplasts and verifying that we are able to reliably detect expression of CP in our protoplast system.

[00101] As shown in ~~Figures 6 and 7~~ Figures 4 and 5, we have successfully infected two host plant species, *Nicotiana benthamiana* and pepper plants. ~~Figure 6~~ Figure 4 shows the infected plants; ~~Figure 7~~ Figure 5 shows a Western blot of upper leaves (not initially infected) analyzed 12 days after inoculation. A1MV CP protein is readily detectable, indicating that we are able to reliably detect expression of CP in infected plant hosts.

[00102] ~~Figure 8~~ Figure 6 shows two TMV-based vectors, 125C and D4, that were engineered to accept insertion of a polynucleotide of interest, following insertion of the polynucleotide (indicated as "foreign gene"). 125C includes TMV coat protein sequences (i.e. sequences extending downstream from nucleotide 5757 of the TMV genome) that contain a *cis* element that may be required for optimal replication. We inserted the gene for human growth hormone (hGH) into each of these vectors between the *Pac*1 and *Xho*1 sites. An AUG was introduced in the 5' primer used to amplify the gene from a plasmid, and the amino acids KDEL were introduced at the 3' end of the coding sequence in order to enhance translation due to retention in the ER. HGH was cloned with and without its native leader sequence; hGH2 lacks the leader and hGH4 includes the leader.

[00103] Primer SR22 (5'-CCG **TTAATTAA**TG TTC CCA ACT ATT CCA; SEQ ID NO:1) was used to clone hGH without its leader, and introducing a *Pac*1 site at the 5' end; primer SR23 (5'-CCG **TTAATTAA**TG GCA ACT GGA TCA AGG; SEQ ID NO:2) was used to clone hGH with its leader. Primer SR24 (5'-CGG **CTC GAG** TTA AAA ACC ACA TGA; SEQ ID NO:3) was used to clone the hGH gene without KDEL and introducing a Xhol site at the 3' end; primer SR25 (5'-CGG CTC GAG TTC ATC TTT AAA ACC TGA TCC; SEQ ID NO:4) was used to clone the gene with KDEL.

Please replace paragraph [00106] with the following amended paragraph:

[00106] *Nicotiana tabacum* suspension protoplasts were harvested at two time points: 24 and 48 hours post inoculation, so that each aliquot contained 500,000 protoplasts. Approximately 2

million protoplasts were used per inoculation of 25uL transcript. The protoplasts were pelleted by centrifugation and the pellet was resuspended in 50 uL buffer (a mixture of Bradley's protein extraction buffer and Laemmli loading buffer). The samples (10 uL) were analyzed by PAGE followed by Western blot hybridization analysis using antiserum to hGH from chicken and anti-chicken IgG conjugated to alkaline phosphatase. Standard hGH was run as a standard. NBT-BCIP was used to develop the blots. ~~Figure 9~~ Figure 7 shows the results of the experiment.

Please replace paragraphs [00108]–[00109] with the following amended paragraphs:

[00108] *Nicotiana benthamiana* plants were also inoculated with *in vitro* transcripts, and the plants were monitored for production of hGH. No signal specific to the protein could be detected at 5 dpi, although at 11 dpi we could detect a signal for hGH in the upper leaves of inoculated plants (~~Figure 10~~ Figure 8).

[00109] We have made constructs to express insulin and proinsulin in plants using our plant virus-based transient expression vectors D4 and 125C. The following primers were used to clone proinsulin into 125C and D4, relying on *Pac*1 and *Xho1* sites for cloning, and adding KDEL at the 3', end of each peptide:

1) *Pac*1 site at 5' end of insulin ORF (B peptide):

SR30 5'-ccg tta att aatg ttt gtt aat caa cat-3' (SEQ ID NO:5)

2) *Xho*1 site at 3' end of A peptide with KDEL

SR31 5'-cgg ctc gag tca gag ttc atc ttt gtt aca gta gtt ctc aag-3' (SEQ ID NO:6)

Please replace paragraphs [00111]–[00114] with the following amended paragraphs:

[00111] D4C3GFP is a TMV-based expression vector that is deficient in CP production (Shivprasad *et al.*, 1999: TTT-GFP) as a result of deletion of the TMV CP coding region and the its replacement with the C3GFP gene, which is placed under the control of the TMVCP subgenomic promoter (see ~~Figure 11b~~ Figure 9b). The C3GFP gene was recloned into D4 by overlapping PCR to eliminate the *Nco*1 and *Xho*1 sites in the C3GFP nucleotide sequence to facilitate further cloning steps. A polylinker *Pst*I-*Not*I-*Xho*I was introduced at the 3'end of C3GFP gene. The PCR product digested with *Pac*I-*Xho*I was cloned into D4 resulting in the version of D4C3GFP shown in ~~Figure 11c~~ Figure 9c.

[00112] The primers we used to modify the C3GFP gene and eliminate *Nco*1 and *Xho*1 sites are:

1) C3GFP .Pac1.For(N)

GGGAG.ATCTTLAATTA.ATGGC.TAGCA.AAGGA.GAAGA.A (SEQ ID NO:7) 36nt

2) C3GFP.Xhol.Rev(N)

CCCCT.CGAGC.GGCCG.CTGCA.GTTAT.TTGTA.GAGCT.CATCC.ATGCC (SEQ ID NO:8) 45nt

3) C3GFP.Ncol.For

GTTCC.CTGGC.CAACA.CTTGT.CAC (SEQ ID NO:9) 23nt

4) C3GFP.Ncol .Rev

TAGTG.ACAAG.TGTTG.GCCAG.GG (SEQ ID NO:10) 22nt

5) C3GFP.Xho1.For

GGACA.CAAAC.TGGAG.TACAA.CTATA (SEQ ID NO:11) 25nt

6) C3GFP.Xhol.Rev

AGTTA.TAGTT.GTACT.CCAGT.TTGTG (SEQ ID NO:12) 25nt

7) (BgIII)-PacI

>AUG...HindIII...NcoI...NdeI...BsrGI...MluI...XhoI...BamHI...MfeI(MunI)...SalI...SacI...TAA< PstI...NotI...XhoI

[00113] Three constructs that contained full-length or portions of the 3'-untranslated region (3' UTR) of A1MV RNA3 were then generated. In each of these constructs, sequences encoding C3GFP under control of the subgenomic TMV CP promoter were present upstream of A1MV RNA3 3'-UTR sequences (either full-length or a portion of the UTR), to allow us to precisely identify the sequences of the A1MV RNA3 3' UTR required for assembly and movement of TMV genomic RNA (either in *trans* or in *cis*). The RNA3 sequences were inserted between the *Not*I and *Xho*I sites of the new D4C3GFP vector as *Not*I-*Sal*I fragments, resulting in the constructs SR25 (nts 1859-1941 of RNA3), SR26 (nts. 1859-1969 of RNA3) and SR27 (nts. 1859-2037 of RNA3, i.e., the entire 3' UTR) (~~Figure 11d~~ Figure 9d). In addition to sequences from the A1MV RNA3 3' UTR, SR25, SR26, and SR27 also include sequences from the TMV 3' UTR (i.e., the UTR from the TMV genomic transcript) downstream of the inserted A1MV sequences. These sequences are TMV nucleotides 6192-6395, as in the D4 construct. The TMV-based viruses (SR25, SR26, and SR27) are defective in long-distance movement because the TMV coat protein is essential for effective phloem-mediated long distance transport and systemic infection of TMV.

[00114] The primers used to generate D4-based constructs with A1MV RNA3 3'-UTR sequences were:

1) SR -52 5' primer with *Xho*l-*Pst*l sites at nt 1859 (plus sense)
5'-CCGCTCGAGCTGCAGTGTACCCCATTAATTTGG-3' (SEQ ID NO:13)
2) SR-53 3' primer at nt 1941 of A1MV RNA3 with *Not*l-*Sal*l sites: minus sense
5'-CGGGTCGACGCGGCCGCGAATAGGACTTCATACCT-3' (SEQ ID NO:14)
3) SR-54 3' primer with *Not*l-*Sal*l sites at nt 1969 of A1MV RNA3: minus sense
5'-CGGGTCGACGCGGCCGCAATATGAAGTCGATCCTA-3' (SEQ ID NO:15)
4) SR-55 3' primer with *Not*l-*Sal*l sites at nt 2037 (minus sense)
5'-CGGGTCGACGCGGCCGCGCATCCCTTAGGGGCATT-3' (SEQ ID NO:16).

Please replace paragraph [0118] with the following amended paragraph:

[00118] Two weeks post infection with A1MV, diffuse GFP fluorescence could be observed in upper leaves of plants infected with SR27 and A1MV but not with SR25 or SR26 and A1MV. ~~Figure 12a~~ Figure 10a shows a picture of a plant that was co-inoculated with SR27 and A1MV. The image (taken under UV light) demonstrates spread of virus into the upper un-inoculated leaves. Fluorescence is caused by the accumulation of GFP. ~~Figure 12c~~ Figure 10c shows the same plant as in ~~Figure 12a~~ Figure 10a, under normal light. ~~Figure 12b~~ Figure 10b (taken under UV light) shows a picture of a plant that was inoculated with SR27 only. Lack of fluorescence in the upper leaves indicates that virus infection was limited to locally inoculated leaves. These results indicate that the CPdeficient TMV-based virus (SR27) containing the GFP transgene moved through the phloem into the upper leaves with the help of A1MV. Generally (e.g., in the absence of trans-complementation from another virus) D4C3GFP only moves into the major veins of the upper leaves 40-45 d.p.i., and SR27 requires similar or even longer periods of time to move into the upper leaves in this system. This result indicates that A1MV can be used as a source for the coat protein that will complement and allow movement of a viral vector that is deficient in one or more coat protein components systemically and provide expression of foreign proteins, including complex proteins such as antibodies. The complementing CP components can be from related (other alfamoviruses, ilarviruses, bromoviruses) or unrelated viruses (TMV, CMV, etc.)